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The endothelial E3 ligase HECW2 promotes endothelial cell junctions by increasing AMOTL1 protein stability via K63-linked ubiquitination

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ABSTRACT

Cell-to-cell junctions are critical for the formation of endothelial barriers, and its disorganization is required for sprouting angiogenesis. Members of the angiomotin (AMOT) family have emerged as key regulators in the control of endothelial cell (EC) junction stability and permeability. However, the underlying mechanism by which the AMOT family is regulated in ECs remains unclear. Here we report that HECW2, a novel EC ubiquitin E3 ligase, plays a critical role in stabilizing endothelial cell-to-cell junctions by regulating AMOT-like 1 (AMOTL1) stability. HECW2 physically interacts with AMOTL1 and enhances its stability via lysine 63-linked ubiquitination. HECW2 depletion in human ECs decreases AMOTL1 stability, loosening the cell-to-cell junctions and altering subcellular localization of yes-associated protein (YAP) from cytoplasm into the nucleus. Knockdown of HECW2 also results in increased angiogenic sprouting, and this effect is blocked by depletion of ANG-2, a potential target of YAP. These results demonstrate that HECW2 is a novel regulator of angiogenesis and provide new insights into the mechanisms coordinating junction stability and angiogenic activation in ECs.

1. Introduction

Angiogenesis is a process that forms new blood vessels and is coordinated by the dynamics of quiescent and activated endothelial cells (ECs). The quiescent state of ECs is maintained until they respond to pro-angiogenic factors, such as VEGF and ANG-2, which promote the liberation of ECs by loosening cell-to-cell junctions [1,2]. Once the junctions become disorganized, subsequent intracellular signaling induces cells to actively proliferate and migrate to form new vessels. Conversely, when ECs come into close contact with each other, actively proliferating ECs form stable intercellular junctions [3,4]. Regulation of endothelial cell-to-cell junctions is critically important in angiogenesis, and incorrect junctional permeability is the primary contributing factor for morbidity and mortality in vascular diseases [5,6].

The angiomotin (AMOT) family is characterized as a group of tight junction proteins that are expressed in ECs and regulate vascular permeability [7–10]. For example, AMOT co-localizes with ZO-1 by cell-to-cell contacts in ECs. In addition, ectopic expression of AMOT results in decreased permeability compared with that in control cells [7], and loss of AMOT-like 1 (AMOTL1) in ECs results in increased vascular permeability in vitro. Further, AMOTL1-transfected morphant zebrafish showed decreased junction stability of stalk cells in the dorsal aorta in vivo [8]. Moreover, AMOT-like 2 (AMOTL2) associated with VE-cadherin for transmission of mechanical force during aortic vessel lumen expansion [11]. A recent study showed that the protein stability of AMOTL1 was required for the formation of the tight junctions [12]. Nevertheless, the mechanism by which AMOTL1 stability is regulated in ECs remains largely unknown.

HECW2, also known as Nedd4-like E3 ubiquitin-protein ligase (NEDL) 2, belongs to the NEDD4 family, which includes nine other members: NEDD4, NEDD4-2, ITCH, SMAD-specific E3 ubiquitin protein ligase (SMURF) 1, SMURF2, WW domain-containing E3 ubiquitin protein ligase (WWP) 1, WWP2, NEDL1, and NEDL2 [13–18]. This family of proteins is characterized by distinct domains: a Ca^{2+}/lipid-binding domain (C2 domain) involved in membrane targeting, 2–4 WW domains that interact with a PPXY motif, and homologous with E6-associated protein C-terminus (HECT)-type ligase domain required for

Abbreviations: AMOT, angiomotin; AMOTL1, angiomotin-like 1; AMOTL2, angiomotin-like 2; YAP, yes-associated protein; ANG-2, angiopoietin-2; VE-cadherin, vascular endothelial cadherin; EPC, endothelial progenitor cell; OEC, outgrowing endothelial cell; HUVEC, human umbilical vein endothelial cell; EC, endothelial cell.

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catalytic activities. This family of E3 ligases regulates diverse cellular processes, such as proliferation, migration, differentiation, invasion, and neuronal cell apoptosis via regulation of target protein interaction with the WW-PPXY domains [19–22].

We recently proposed a molecular mechanism involving the YAP in vascular ECs. YAP is a transcriptional co-activator that plays a role in maintaining cellular homeostasis [23]. YAP was found to be expressed in the angiogenic front region where vessels were sprouting and regulated by the VE-cadherin-mediated PI3K/AKT pathway. Once YAP was activated by disruption of junctions, YAP translocated into the nucleus and induced ANG-2 expression, thereby promoting angiogenic sprouting in vitro [24].

Here we investigate the role of HECW2 in ECs. We demonstrate that HECW2 enhances endothelial cell-to-cell junctions through the regulation of AMOTL1 protein stability via lysine 63-linked polyubiquitination. Furthermore, depletion of HECW2 stimulates YAP translocation into the nucleus, thereby promoting EC sprouting via increased ANG-2 expression.

2. Materials and methods

2.1. Cell culture and antibodies

Human umbilical vein ECs (HUVECs) were isolated from human umbilical cord veins using collagenase, as previously described [25], and cells (between passage 2 and 7) were cultured on 2% gelatin-coated dishes using M199 medium (Invitrogen, Carlsbad, CA), containing 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor (R&D systems, Minneapolis, MN, USA), and 5 U/ml heparin. HUVECs were grown at 37 °C in a humidified 95%/5% (vol/vol) mixture of air and CO_2. The following antibodies were used: HECW2 and AMOTL1 (Atlas Antibodies, Stockholm, Sweden); ANG-2 (R&D systems, Minneapolis, MN, USA); YAP, LAMIN A/C, GFP, GAPDH, and β-ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-YAP (S127; Cell Signaling, Danvers, MA, USA); and angiopoetin-2 (R&D systems, Minneapolis, MN, USA).

2.2. Plasmids

The HECW2 and AMOTL1 open-reading frame (ORF)-containing plasmids Flag-HECW2 WT, FLAG-AMOTL1 and 2, and HA-HECW2 WT were generated in the Vascular genomics laboratory, Yonsei University. The AMOT130 ORF-containing plasmid HA-AMOT130 was purchased from Addgene (Cambridge, MA, USA), and AMOT130 ORF was subcloned into a FLAG vector. The HECW2 C1540A mutant was generated by site-directed mutagenesis. HECW2 deletion mutants were generated by polymerase chain reaction (PCR). V5-Ub was kindly provided by Dae-Won Kim (Yonsei University). pHM6-HA-Ub and mutants were kindly provided by Jaewhan Song (Yonsei University). pEGFP-C2 was used as a transfection control (Clontech, San Diego, CA).

2.3. Transfection of siRNA

HUVECs were transfected with scrambled and HECW2 siRNAs using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) for 3 h. Efficiency of siRNA was assessed by RT-PCR and Western blot 48 h after transfection. siRNA targeting HECW2 was purchased from Dharmacon Inc. (siGENOME HUMAN (MQ-007192-00-0005)) (Lafayette, CO, USA).

2.4. Immunoprecipitation

HUVECs were plated on 100-mm-diameter dishes. The cells were lysed with lysis buffer [150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.1% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture]. After centrifugation for 10 min at 13,000 rpm, the supernatant was removed, and the cell lysate was incubated with indicated antibodies for 12 h at 4 °C. The cells were incubated with protein G-Sepharose beads (GE Healthcare, Buckinghamshire, United Kingdom) for 2 h at 4 °C. The samples were then centrifuged and washed three times with lysis buffer and added to 2× sample buffer and then boiled.

2.5. Ubiquitination assay

HEK293T cells were transfected with plasmids expressing FLAG-AMOTL1, HA-HECW2, and HA-tagged Ub. After 24 h of transfection, the cells were lysed and harvested in phosphate-buffered saline (PBS) containing 10 nM NEM to prevent deubiquitination. Cells were lysed in 1% SDS by boiling for 10 min, followed by dilution to 0.1% SDS by the addition of lysis buffer, protease inhibitors, and NEM. Lysed samples were immunoprecipitated with a FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA), followed by Western blotting.

2.6. Fibrin gel beads sprouting assay

HUVECs were mixed with dextran-coated Cytodex 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a concentration of 400 HUVECs per bead in 1 ml of EGM-2 medium (Clonetics, Walkersville, MD, USA). Beads with cells were shaken gently every 20 min for 4 h at 37 °C and 5% CO_2. After incubation, mixtures of beads and cells were transferred to a 25-cm^2 tissue culture flask (BD Biosciences, Bedford, MA, USA) and incubated in 12–16 h in 5 ml of EGM-2 at 37 °C and 5% CO_2. The following day, mixtures of beads and cells were washed three times with 1 ml EGM-2 and were resuspended at a concentration of 250 cell-coated beads/ml in 2 mg/ml fibrinogen (Sigma) with 0.15 U/ml of aprotinin (Sigma) at a pH of 7.4. Five hundred microliters of fibrinogen/bead solution was added to 1 unit of thrombin (Sigma) in 1 well of a 24-well tissue culture plate. The fibrinogen/bead solution was allowed to clot for 5 min at room temperature and for 20 min at 37 °C and 5% CO_2. One milliliter of EGM-2 (which contains 10% FBS) was added to each well and was equilibrated with the fibrin clot for 30 min at 37 °C and 5% CO_2. The medium was removed from the well and replaced with 1 ml of fresh medium with or without additional growth factors. Twenty thousand skin fibroblasts were plated on the clot and the medium was changed every other day. Bead assays were monitored for 10 days.

2.7. Immunofluorescence staining

Scramble or HECW2 siRNA-transfected HUVECs cultured on coverslips in a 12-well plate were fixed with 4% paraformaldehyde ((PFA)-PBS) at room temperature for 20 min and were permeabilized with 0.1% Triton X-100 in PBS with Tween-20 for 15 min. After blocking by addition of 1% bovine serum albumin in PBS with Tween 20 for 1 h, cells were incubated with primary antibodies in blocking buffer at 4 °C overnight. After washing with PBS, cells were incubated with Alexa Fluor 488 goat *anti*-mouse for 30 min at room temperature. Nuclei were stained with 4, 6 diamidino-2-phenylindole (DAPI; Molecular Probes, Carlsbad, California, USA). Cells were mounted, and images were acquired by a laser scanning confocal microscope (LSM 700 META, Carl Zeiss). For quantification, at least 500 cells were counted in each sample.

2.8. Lentivirus production

Human TRC HECW2 shRNA constructs were purchased from Dharmacon (RHS4533-EG57520). To produce the lentivirus, pLenti-shHECW2 and 2nd generation packaging DNA were transfected into HEK293T cells. After 48 h, the supernatant was harvested and filtered using a 0.45 μm filter to remove cellular debris. Lentivirus was titrated to 10^7 TU per ml.

2.9. FITC-dextran permeability assay

HUVECs were grown until confluent on 0.1% gelatin-coated Transwell™ filters (Corning Costar). Cells were serum-starved in endothelial serum-free medium for 2 h. FITC-dextran (1 mg/ml; Sigma) was added to the upper compartment. Absorbance from the lower chamber solution was measured at 492 nm excitation and 520 nm emission in a FLUOstar Omega.

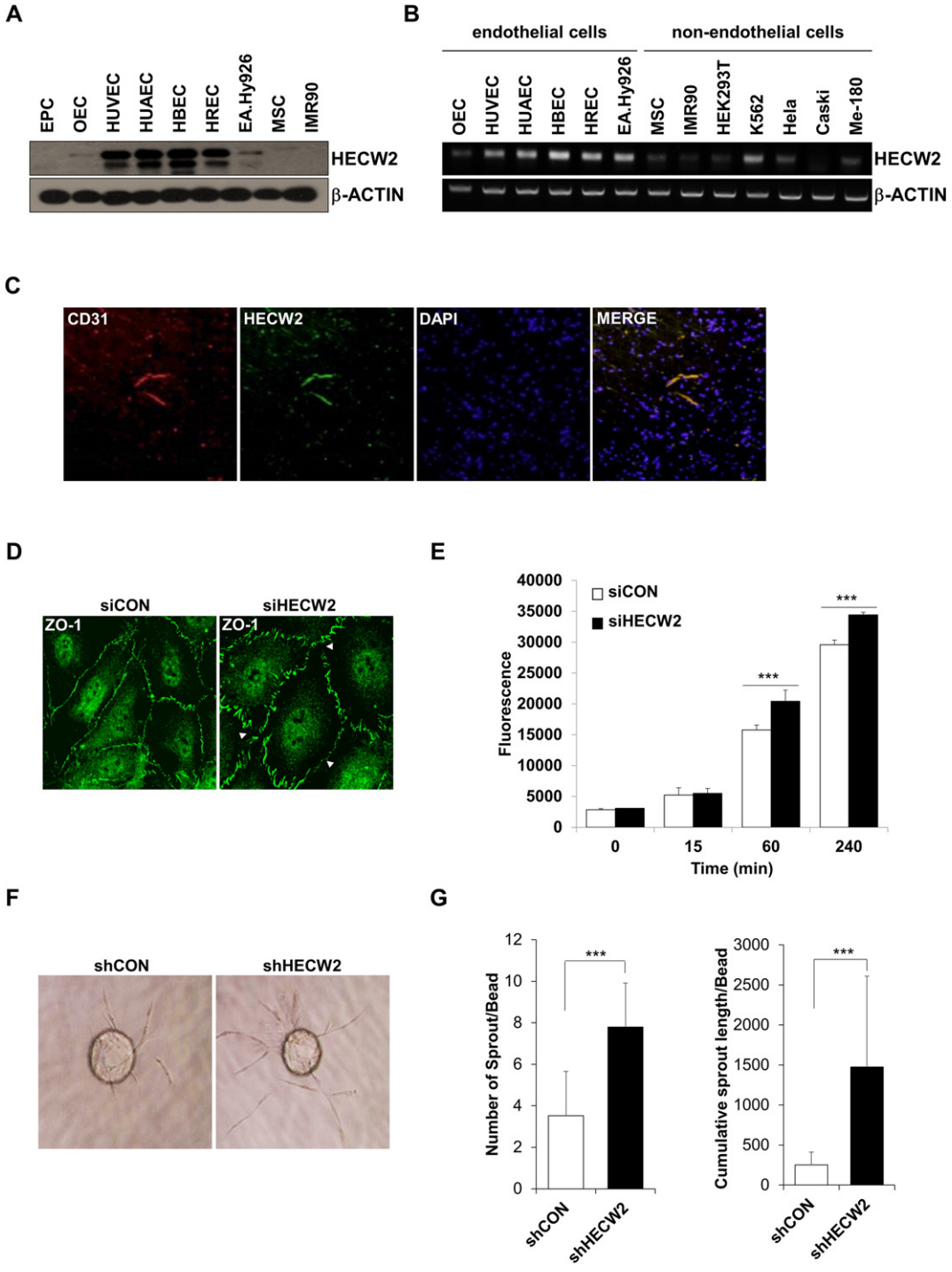

Fig. 1. HECW2 regulates EC junction stability and sprouting angiogenesis. (A, B) Various human endothelial and non-endothelial cell lines were analyzed by Western blot or RT-PCR. The cell lysates were immunoblotted with *anti*-HECW2 and *anti*-beta-ACTIN antibodies. cDNA was synthesized from mRNA extracted from the cells and was analyzed using primers for HECW2 and beta-ACTIN (C) Immunofluorescence staining of HECW2 in human brain sections was performed using *anti*-CD31 and *anti*-HECW2 antibodies. (D) Immunofluorescence analysis of EC junctions in HECW2 siRNA-treated HUVECs was performed using antibodies against ZO-1. The arrows indicate disrupted junctions. (E) The paracellular permeability of HUVECs increased with HECW2 depletion compared with that of cells transfected with control siRNA. siRNA-transfected HUVECs were seeded at confluence on a well insert. The permeability of the monolayer to 70 kDa FITC-dextran was evaluated at 0, 15, 60, and 240 min by measuring fluorescence of the lower well. (F) After HUVECs were infected with control shRNA (shCON) or HECW2-targeting shRNA, the bead-sprouting assay was performed and was replenished with 2% EGM medium every other day. (G) The number of sprouts or cumulative sprout length derived per bead was counted after 6 days of incubation. Data are shown as mean ± SEM. of values from a representative experiment. $^{***}p < 0.0001$ versus shRNA-infected CON cells. (two-tailed Student's *t*-test).

2.10. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses involved Student's *t*-test and one-way analysis of variance (ANOVA). *p*values of 0.05 were considered significant.

3. Results

3.1. HECW2 is highly expressed in endothelial cells

Using Affymetrix oligonucleotide arrays, we have identified various genes that are regulated during EC differentiation from endothelial progenitor cells (GEO Accession No. GSE12891) [26]. In this study, we focused on ubiquitin E3 ligases because regulation of junction protein stability is important in maintenance of vascular integrity. We examined HECT ubiquitin ligase family member expression in ECs. Among them, compared with other gene members, HECW2 showed relatively specific expression in differentiated ECs (Supplementary Fig. 1A and B). RT-PCR and Western blot analyses also confirmed that compared with early stage EPCs, HECW2 is highly expressed in OECs and HUVECs (Fig. 1A and B). Furthermore, immunostaining of human brain sections revealed that HECW2 co-localized with CD31, a well-known EC marker (Fig. 1C), suggesting that HECW2 may play a role in EC function.

3.2. HECW2 depletion leads to disruption of junctions and promotes angiogenic sprouting

To understand the role of HECW2 in ECs, we silenced endogenous HECW2 gene using siRNA. First, we examined whether siRNA of HECW2 effectively targeted the HECW2 gene in HUVECs. We used four different siRNAs for HECW2. All siRNAs showed high efficiency (Supplementary Fig. 3A). To reduce cytotoxicity and off-target effects, we pooled the siRNAs. Then, using siRNA-targeted knockdown of HECW2, we evaluated the effects of this protein on EC properties important for cellular functions, such as cell junction, permeability, and sprouting. Knockdown of HECW2 showed breakage of junctions and increased EC permeability. Scrambled siRNA-transfected cells showed well-organized junctions, whereas the junctions in HECW2-silenced cells were disrupted (Fig. 1D). Permeability analysis using transwell revealed that HECW2 siRNA-transfected cells showed increased permeability (Fig. 1E). These data suggest that HECW2 may play a role in EC junction stability. To further understand the role of HECW2, we examined EC sprouting using a three-dimensional fibrin gel bead assay. Knockdown of HECW2 using lentiviruses increased EC sprouting (Fig. 1F and G). Taken together, these data indicate that HECW2 may be involved in the regulation of junction stability and angiogenic activation.

3.3. HECW2 interacts with AMOTL1

HECT-type E3 ligases mainly regulate target proteins through interaction with their WW-PPxY domain. Many groups have identified WW domain-interacting proteins through cDNA libraries and yeast two-hybrid analyses [27–30]. Among various candidates, we selected the AMOT family because of functional studies that establish their role in angiogenesis [31]. The AMOT family has three members: AMOT, AMOTL1, and AMOTL2. The AMOT family is a group of angiogenic regulators which are involved in cell polarity, junction regulation, and sprouting angiogenesis [8]. All members are capable of interacting with the HECW2 protein (Supplementary Fig. 2A and B). However, AMOT and AMOTL1, but not AMOTL2, were stabilized by HECW2 (Supplementary Fig. 2C). Because AMOT expression was barely detected in ECs, we focused on AMOTL1 (Supplementary Fig. 2D). To test whether HECW2 interacts with AMOTL1 in ECs, HUVECs cell lysates were analyzed using IP-Western blotting. Western blotting analysis revealed that HECW2 specifically interacts with AMOTL1 (Fig. 2A–D). Because HECW2 has two WW domains and AMOTL1 contains two WW binding proline-rich (P-Y) motifs, the relative abilities of the WW domains of HECW2 to bind AMOTL1 were determined. We generated seven HECW2 mutants that were distinguishable by mutations in their WW domains to identify the interaction motif of AMOTL1. HECW2 WT and four mutants (807-1572, 807-1012, 324-1012, and 1-1213) contained the WW domain while the rest of the mutants do not (Fig. 2E). HECW WT and WW-containing mutants interacted with AMOTL1 (Fig. 2F). These data revealed that the WW domain of HECW2 contributed to the interaction with AMOTL1.

3.4. HECW2 stabilizes AMOTL1 through lysine 63-linked polyubiquitination

A previous study demonstrated that NEDD4.2, one of HECT E3 ligases, targeted AMOTL1 for ubiquitin-dependent degradation [12]. Unlike NEDD4.2, HECW2 targeted AMOTL1 and promoted its stability. HECW2 and AMOTL1 were transfected into HEK293T cells in a concentration-dependent manner. Overexpressed HECW2 induced stabilization of exogenous AMOTL1 (Fig. 3A). As described above, HECW2 contains a C-terminal catalytic HECT domain with a conserved catalytic cysteine located in its HECT domain. When HECW2 C1540 was mutated to an A residue, the E3 ligase activity was completely abolished [32]. To examine whether AMOTL1 was stabilized by ubiquitination, we generated a dominant negative mutant in which alanine was substituted for the 1540th cysteine residue. This C1540A mutant lost its ability to stabilize AMOTL1 (Fig. 3B). Because HECW2 interacted with AMOTL1, we investigated whether the role of HECW2 in AMOTL1 protein stability was to act as an ubiquitin ligase. Thus, we performed an in vivo ubiquitination assay to assess whether HECW2 induces AMOTL1 ubiquitination. Ectopic expression of HECW2 induced AMOTL1 polyubiquitination but the C1540A mutant did not (Fig. 3C and Supplementary Fig. 4). Polyubiquitination leads to different functional consequences depending on the polyubiquitin chains linked through K48, K63, or other K residues of Ub. Although K48-linked polyubiquitin chains target substrates in the 26S proteasome for degradation, K63-linked polyubiquitin chains initiate non-degradation signaling, including stabilization and translocalization [33–35]. To further confirm this result, two different ubiquitin mutants K48R and K63R were used. Consistently, HECW2-mediated AMOTL1 polyubiquitination was supported by WT and K48R Ub, but not by K63R Ub (Fig. 3D). These results clearly indicate that HECW2 ubiquitinates AMOTL1 with K63-linked polyubiquitin chains. Furthermore, we clarified whether subcellular localization of the stabilized AMOTL1 is altered by HECW2. First, we examined the localization of AMOTL1 using an antibody against AMOTL1 and also confirmed whether siRNA of AMOTL1 effectively targeted the AMOTL1 gene in HUVECs. Western blot and immunofluorescence analysis revealed that AMOTL1 was localized in the EC junction area and that siAMOTL1-transfected HUVECs showed remarkably decreased AMOTL1 expression (Supplementary Fig. 5A and B). Overexpressed HECW2 WT but not the C1540A mutant induced AMOTL1 localization to the tight junction area (Fig. 3E). These data suggest that, in contrast to NEDD4.2, HECW2 promoted AMOTL1 protein and tight junction stability.

3.5. Depletion of HECW2 decreased AMOTL1 protein stability in ECs

A previous study demonstrated that AMOTL1 regulates EC junctional stability [8]. To confirm this, we performed immunostaining using antibodies against ZO-2 and VE-CADHERIN, which are the well-known markers for tight junctions and adherent junctions, respectively. siAMOTL1-transfected HUVECs showed disrupted VE-CADHERIN and ZO-2 staining as indicated by white arrows (Supplementary Fig. 6A). To clarify whether stabilization of AMOTL1 by HECW2 is required for EC tight junctions, we silenced HECW2 using siRNA in HUVECs. Through RT-PCR and Q-PCR analysis, we found that siHECW2 transfection did not alter AMOTL1 mRNA expression (Fig. 4A). However, knockdown of HECW2 markedly decreased AMOTL1 protein levels in HUVECs (Fig. 4B). In general, as K48-linked polyubiquitination of substrates leads to

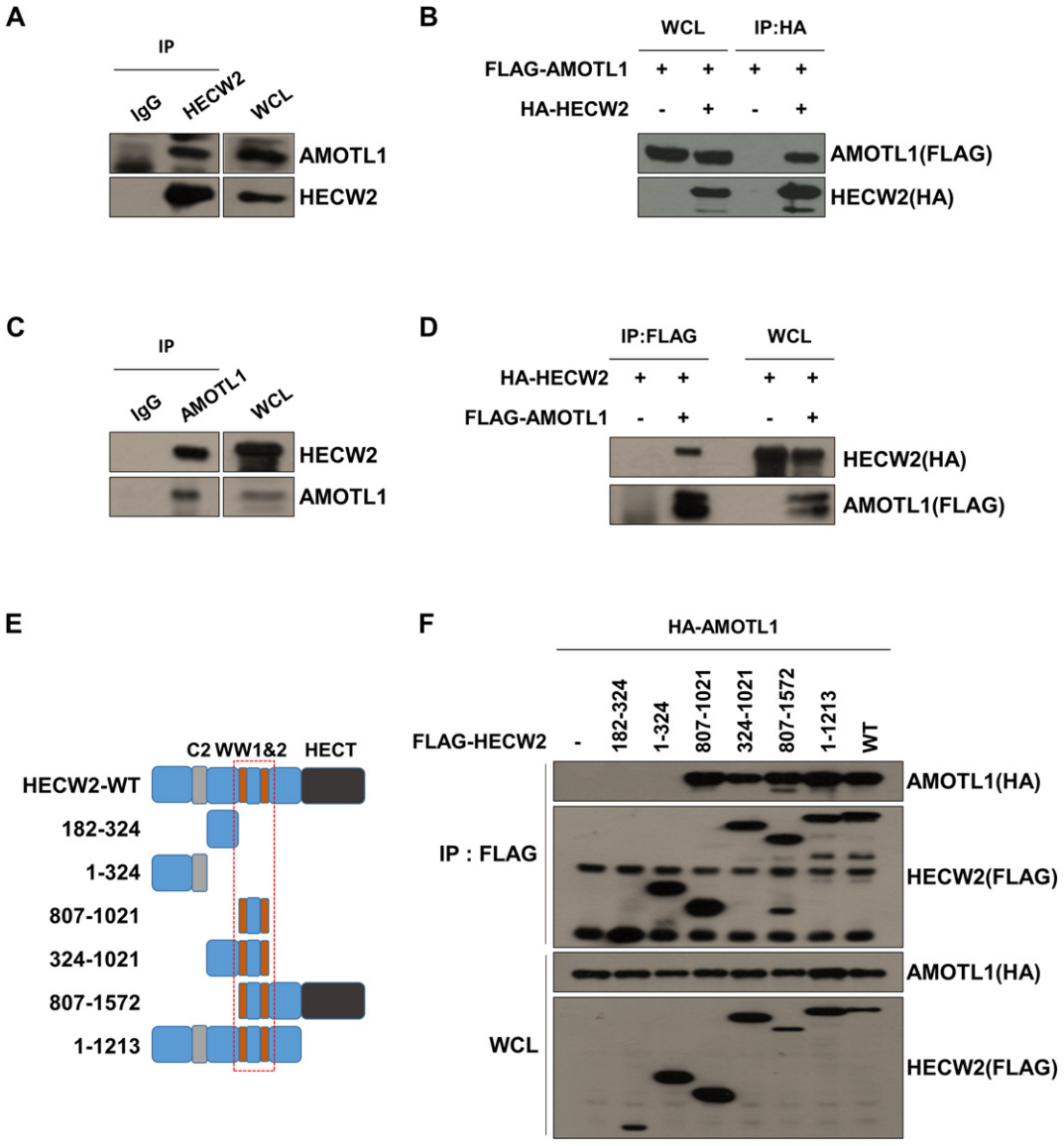

Fig. 2. HECW2 interacts with AMOTL1. (A) Endogenous HECW2 binds to AMOTL1. HUVEC cell lysates were immunoprecipitated using the *anti*-HECW2 antibody, and immunoprecipitated proteins were analyzed using the *anti*-AMOTL1 antibody. (B) Exogenous HECW2 interacts with exogenous AMOTL1. Plasmids expressing HA-HECW2 alone or with FLAG-AMOTL1 were transfected into 293T cells. Whole-cell lysates (WCL) were immunoprecipitated with an *anti*-HA antibody, and both immunoprecipitates and WCLs were immunoblotted using *anti*-HA and *anti*-FLAG antibodies. (C) Endogenous AMOTL1 binds to HECW2. HUVEC cell lysates were immunoprecipitated using the *anti*-AMOTL1 antibody, and immunoprecipitated proteins were analyzed using the *anti*-HECW2 antibody. (D) Exogenous AMOTL1 interacts with exogenous HECW2. Plasmids expressing FLAG-AMOTL1 alone or with HA-HECW2 were transfected into 293T cells. Whole-cell lysates (WCL) were immunoprecipitated with an *anti*-FLAG antibody, and both immunoprecipitates and WCLs were immunoblotted using *anti*-HA and *anti*-FLAG antibodies. (E) Schematic of a series of HECW2-deletion mutants. (F) FLAG-HECW2-deletion mutants and HA-AMOTL1 were transfected into HEK293T cells. Cell lysates were immunoprecipitated with *anti*-FLAG antibody. Both the lysate and immunoprecipitates were immunoblotted using the *anti*-FLAG and *anti*-HA antibodies.

proteasomal degradation and K63-linked polyubiquitination affects localization, signaling, and stabilization, we first tested whether HECW2 affects any of these characteristics of AMOTL1 using siRNA-knockdown of HECW2. We checked the stability of the AMOTL1 protein after CHX treatment in siCON- or siHECW2-transfected HUVECs. Depletion of HECW2 decreased the half-life of the AMOTL1 protein, confirming the importance of HECW2 in regulating AMOTL1 stability (Fig. 4C). Knockdown of HECW2 decreased AMOTL1 localization to tight junction areas (Fig. 4D). Therefore, these results suggest that depletion of HECW2 diminishes cell-to-cell junctions via AMOTL1 destabilization.

3.6. HECW2 depletion induced translocation of YAP into the nucleus

AMOTL1 is a negative regulator of YAP that acts by triggering its phosphorylation via LATS1/2. Once YAP is dephosphorylated by AMOTL1 depletion, cytoplasmic YAP moves into the nucleus to regulate downstream target genes [36,37]. We hypothesized that HECW2 may be involved in the translocation of YAP via AMOTL1 stabilization in ECs. To test this hypothesis, we examined YAP immunostaining in AMOTL1- or HECW2-depleted ECs. AMOTL1 or HECW2 depletion increased nuclear YAP compared with that in the control (Fig. 5A and Supplementary Fig. 6B). We performed fractionation of ECs to determine YAP localization and analyzed the fractions by Western blotting analysis. Knockdown of or HECW2 showed decreased cytosolic YAP and increased nuclear YAP, indicating that HECW2 altered YAP cellular localization by regulating its phosphorylation. We also found that AMOTL1 levels were decreased in cytosolic fractions, strongly suggesting that the expression level of AMOTL1 may be closely related to translocation of YAP (Fig. 5B and C and Supplementary Fig. 6C).

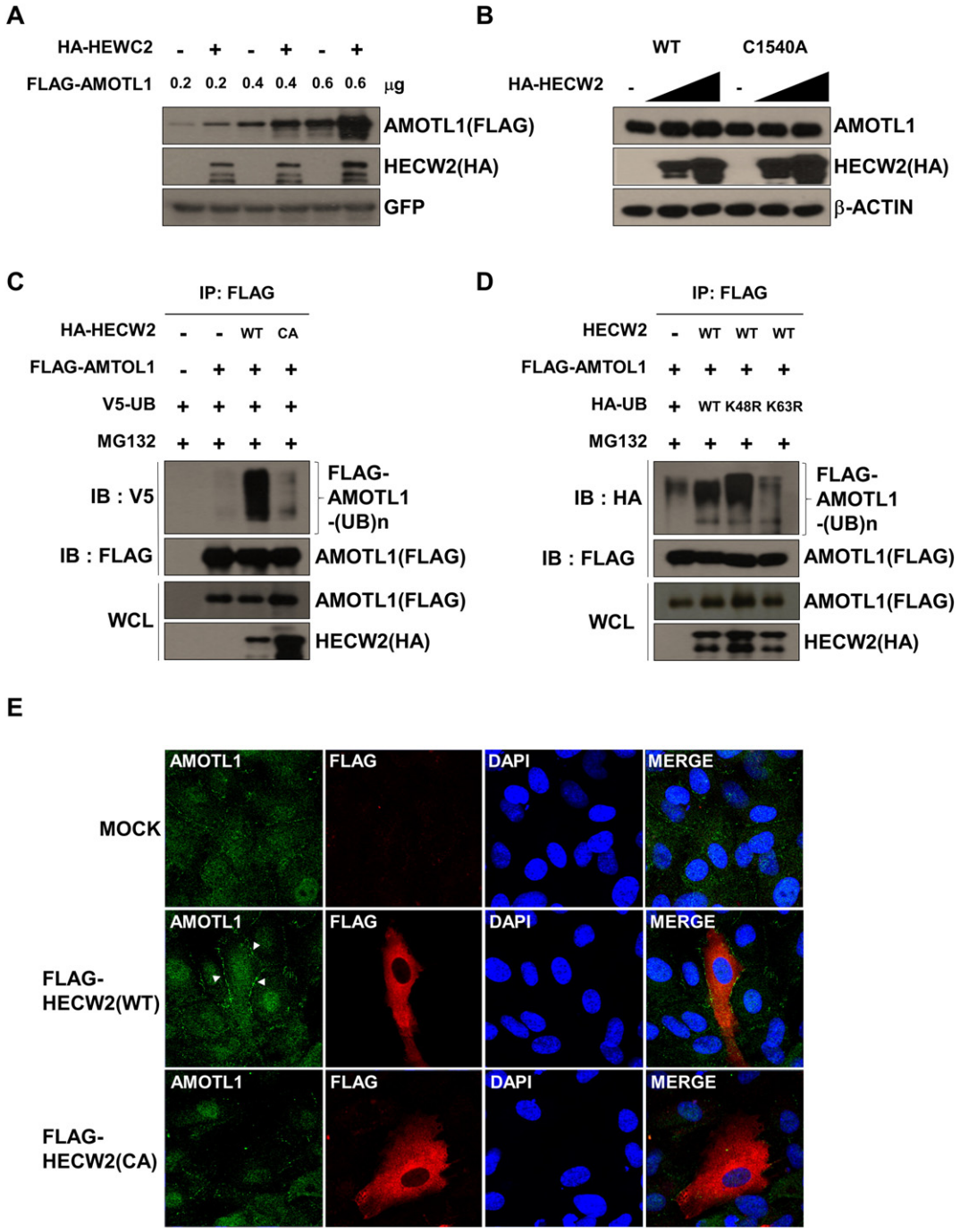

Fig. 3. HECW2 stabilizes AMOTL1 protein by K63-linked ubiquitination. (A) Exogenous HECW2 upregulates exogenous AMOTL1 protein levels. A combination of plasmids expressing HA-HECW2, Flag-AMOTL1, and GFP were transfected into 293T cells. WCL were analyzed by immunoblotting. GFP was used as a control. (B) Exogenous HECW2 WT upregulates endogenous AMOTL1 protein levels but exogenous HECW2 C1540A mutant does not. Extracts of 293T cells transfected with plasmids expressing HECW2 WT or the C1540A mutant were analyzed by immunoblotting with *anti*-HA, *anti*-AMOTL1, and an *anti*-beta-ACTIN antibody. (C) The C1540A mutant cannot induce polyubiquitination of AMOTL1. Combinations of plasmids were transfected, as indicated, into 293T cells with MG132 for 6 h before harvest. Ubiquitinated forms of AMOTL1 were detected using *anti*-V5 antibody (upper panel). (D) HECW2 utilized K63-linked polyubiquitination of AMOTL1. Different types of ubiquitin mutant were transfected into 293T cells with a combination of plasmids, as indicated above. The cell lysate was immunoprecipitated using an antibody against FLAG and was immunoblotted with an antibody against HA to detect ubiquitination. (E) HECW2 overexpression promotes AMOTL1 in endothelial cell junctions. HUVECs were infected with Lentivirus MOCK, HECW2 WT, and HECW2 C1540A. Infected HUVECs were stained with the *anti*-AMOTL1 and *anti*-FLAG antibodies. The arrows indicate tight junction areas.

3.7. Endothelial sprouting induced by HECW2 depletion is mediated by ANG-2

As we showed in Fig. 1F, HECW2-depleted cells showed increased angiogenic sprouting. Interestingly, AMOTL1-depleted cells also exhibited increased angiogenic sprouting (Supplementary Fig. 7A and B). Our previous study revealed that YAP activation induced by junction destabilization increased ANG-2 expression, thereby promoting angiogenic sprouting. To investigate whether HECW2 is also involved in YAP-mediated ANG-2 pathway, we examined ANG-2 levels in

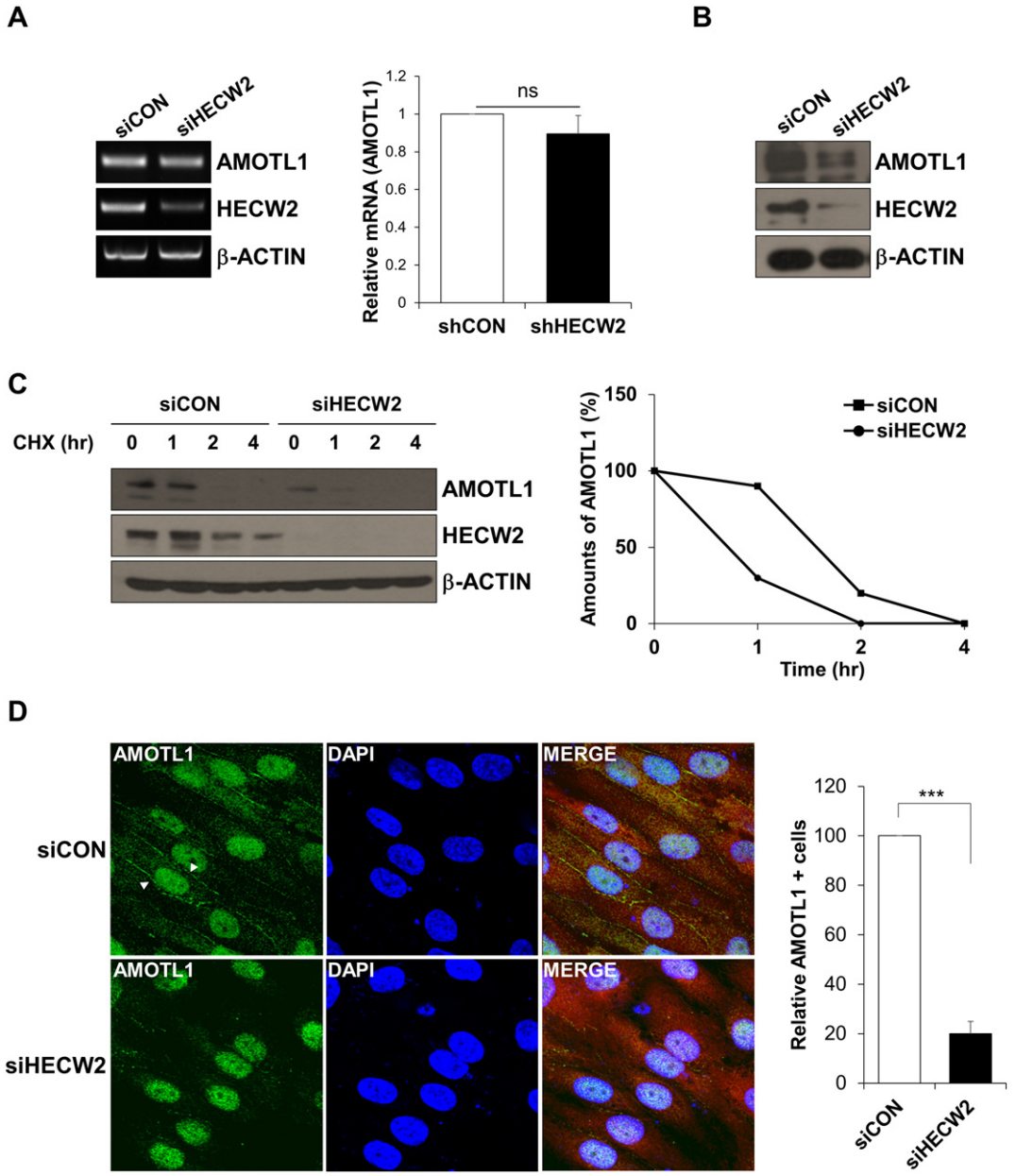

Fig. 4. Loss of HECW2 decreases AMOTL1 protein stability in HUVECs. (A) siCON- or siHECW2-transfected HUVECs were analyzed by RT-PCR (left) and Q-PCR (right). (B) siCON- or siHECW2-transfected HUVECs were analyzed by Western blotting analysis. (C) HUVECs were transfected with the control and HECW2 siRNAs followed by treatment with or without CHX and were then lysed and immunoblotted with *anti*-HECW2, *anti*-AMOTL1, and *anti*-beta-ACTIN antibodies. (D) Loss of HECW2 decreases AMOTL1 in endothelial cell junctions. HUVECs were transfected with control or HECW2 siRNA. Transfected HUVECs were stained with *anti*-AMOTL1 antibody. The arrows indicate tight junction areas. The number of AMOTL1-positive cells was counted in siCON- or siHECW2-transfected HUVECs.

HECW2-depleted ECs. Knockdown of HECW2 showed increased levels of both ANG-2 protein and RNA (Fig. 6A and B). Moreover, knockdown of AMOTL1 showed increased ANG-2 expression (Supplementary Fig. 7C). The increase in sprouting following HECW2 depletion was blocked by ANG-2 knockdown (Fig. 6C and D). Therefore, these data implicate HECW2 as an important regulator of AMOTL1-YAP pathway-mediated regulation of angiogenesis.

4. Discussion

Here we identify HECW2 as a novel E3 ligase in ECs and demonstrate that it plays regulatory roles in EC junction stability and angiogenic activation. A previous study has shown that HECW2 enhances p73 protein stability via polyubiquitination in non-ECs and increases its transcriptional activity [13]. p73 is important in EC differentiation and vasculogenesis by regulating VEGF and TGFβ signaling. p73-deficiency results in reduced VEGF and TGFβ signaling and impaired angiogenic sprouting in mESCs [38,39]. Based on a study on p73 and HECW2, HECW2 deficiency is supposed to show impaired angiogenesis. However, HECW2 did not change p73 protein stability in ECs. On the other hand, HECW2 deficiency showed promoted sprouting angiogenesis and this data suggested that p73 and HECW2 deficiency showed opposite phenomena.

Cell-to-cell junctions in ECs maintain vessel integrity, and junction stability is tightly regulated by post-translational modifications, such as ubiquitination [40,41]. To date, over 30 HECT-type ubiquitin ligases

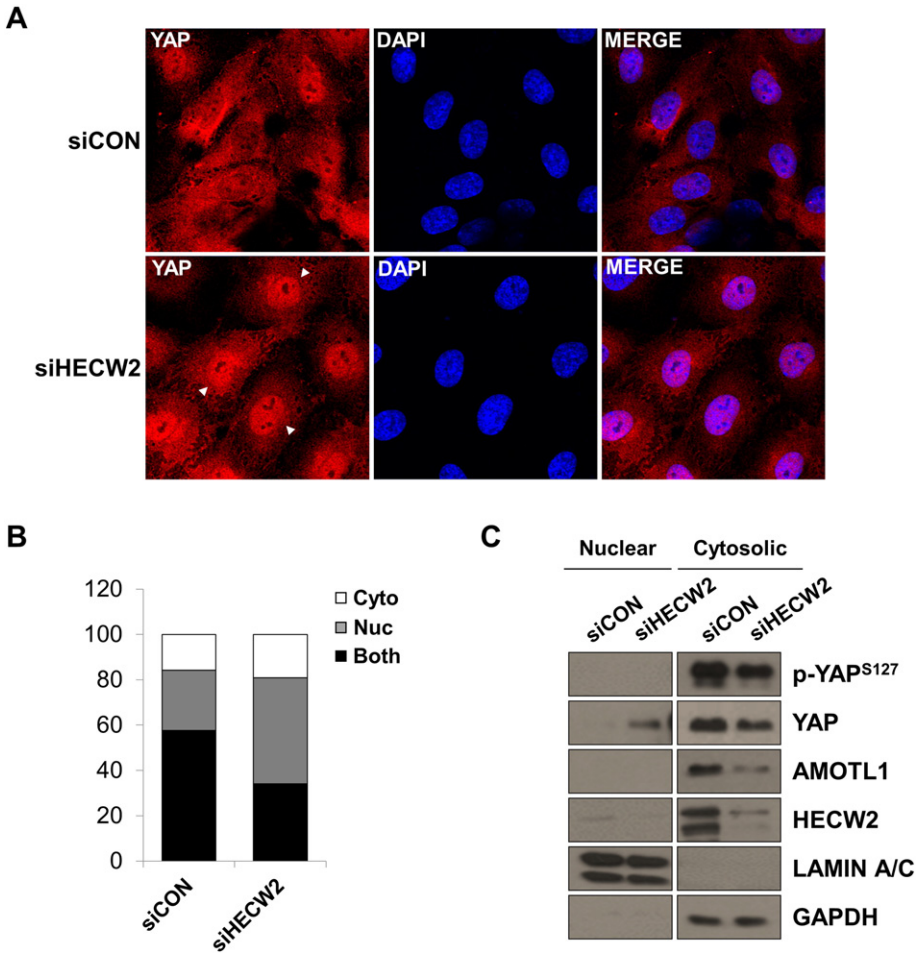

Fig. 5. HECW2 depletion induced translocation of YAP into the nucleus. (A) Immunofluorescence analysis of YAP in HECW2 siRNA-treated HUVECs was conducted using antibodies against YAP. The arrows indicate nuclear YAP. (B) Intracellular localization of YAP was quantified. (C) The cytosolic or nuclear fractions of cell lysates were immunoblotted with *anti*-HECW2, AMOTL1, YAP, and phospho-YAP (S127) antibodies. *Anti*-LAMIN A/C and GAPDH were used as loading controls.

are known and are reported to participate in various cellular processes [19,42]. Among them, only a few have been studied in the context of EC junction stability. ITCH (also known as AIP) is an E3 ligase of occludin. The ubiquitin-induced trafficking of occludin promotes VEGF-induced vascular permeability [43]. Another E3 ligase, NEDD4.2, targets AMOTL1 for ubiquitin-dependent degradation, leading to its mislocalization from the tight junctions [12]. On the contrary, we identify HECW2 as another key E3 ligase for AMOTL1. However, unlike NEDD4.2, HECW2 increased protein stability of AMOTL1 via K63-linked ubiquitination and increased its localization to junction areas in HUVECs. HECW2-depleted cells showed mislocalization of AMOTL1 away from the tight junctions. Although the molecular mechanisms of regulation of AMOTL1 by NEDD4.2 and HECW2 in ECs are still under investigation, our findings suggest that there is an inverse regulation of AMOTL1 protein stability by E3 ligases.

Recent studies have shown that AMOT and AMOTL1 exhibited similar functions by interacting with and regulating tight junction proteins, including ZO-1 and YAP [44,45]. AMOT-deficient mice showed dilated vessels in the brain, resulting in severe lethality between E11 and E11.5; only 5% survived without any vascular defects [46]. Thus, other family members may compensate for loss of AMOT. Because of their conserved domains and known functions, AMOTL1 was the most likely candidate [8]. We observed that HECW2 interacted with all AMOT family members, but AMOT expression was not detected in HUVECs. Thus, we concluded that junction destabilization by HECW2 depletion might be mediated by AMOTL1.

AMOTL1 was suggested to be an upstream negative regulator of YAP through direct binding, leading to tight junction localization and cytosolic retention of YAP. Knockdown of the AMOT family members in MDCK cells leads to loss of tight junction localization and nuclear accumulation of YAP and induction of YAP target gene expression [45]. YAP is a transcriptional co-activator of the Hippo pathway, which is tightly regulated by cell-to-cell contacts [47–49]. We have previously reported that YAP directly regulates the transcription level of ANG-2 in sprouting angiogenesis [24]. However, the underlying mechanism and the genes involved in regulation of YAP in ECs remain unclear. We found that YAP was translocated into the nucleus by depletion of HECW2 and that this nuclear YAP augmented ANG-2 expression, thereby promoting EC sprouting. Although the mechanism by which HECW2 is regulated in ECs is still being investigated, our current study provides new insights into the regulatory mechanisms of YAP-mediated sprouting angiogenesis.

In conclusion, here we introduce an important mechanism connecting EC junction stability and sprouting angiogenesis. The known role of the AMOTL1-YAP pathway is highly consistent with our finding that depletion of HECW2 increased ANG-2 expression. We show for the first time the mechanism by which HECW2 regulates the AMOTL1-YAP pathway in ECs. Thus, our findings provide important insights into the role of HECW2, as an E3-ligase, in regulating EC junction stability.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2016.07.015.

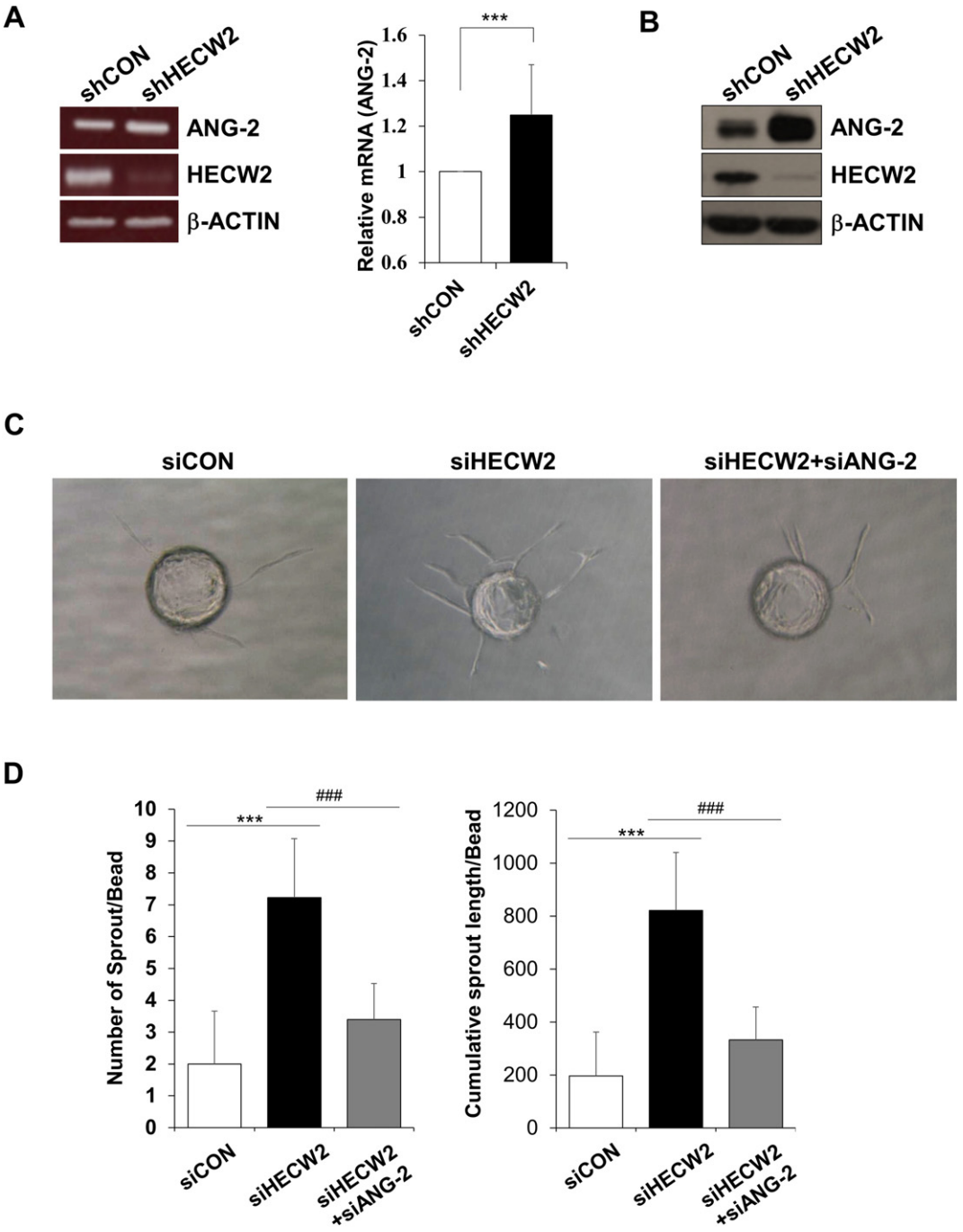

Fig. 6. Endothelial sprouting induced by HECW2 depletion is mediated by ANG-2. (A) The mRNA expression of ANG-2 in shCON- or shHECW2-infected HUVECs was analyzed by RT-PCR (left) and Q-PCR (right). (B) The protein expression of ANG-2 in shCON- or shHECW2-infected HUVECs was analyzed by Western blotting analysis. Data are shown as mean ± SEM of values from a representative experiment. (C) After HUVECs were transfected with siCON or siHECW2 with or without siANG-2, respectively, the bead-sprouting assay was performed and was replenished with 2% EGM medium every other day. (D) The number of sprouts or cumulative sprout length derived per bead was counted after 4 days of incubation. Data are shown as mean ± SEM of values from a representative experiment. $^{***}p < 0.0001$ vs siCON transfected cells; $^{\#\#\#}p < 0.0001$ vs siHECW2 + siANG-2-transfected cells (two-tailed Student's *t*-test).

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